

App'n Serial No.: 10/716,480

4

Att'y Docket No: US-102

**IN THE SPECIFICATION:**

*Kindly amend the specification as follows, in accordance with 37 C.F.R. § 1.121:*

Please replace paragraphs [0029], [0088], [0089], [0092], [0101], [0115], [0117], [0122] and [0123] in the specification with the following paragraphs:

[0029] In the present invention, the methanol-assimilating bacterium, that is, methylotroph, means a bacterium which can grow by utilizing methanol as a major carbon source, ~~and in which the function of the LysE protein is expressed when the DNA of the present invention is introduced.~~ Specific examples include *Methylophilus* bacteria such as *Methylophilus methylotrophus* and *Methylobacillus* bacteria such as *Methylobacillus glycogenes* and *Methylobacillus flagellatum*.

[0088] Specifically, pRS was constructed as follows. The pVIC40 plasmid was digested with *EcoRI* and added with a phenol/chloroform solution and mixed with it to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNAs were collected by ethanol precipitation and separated on 0.8% agarose gel. A DNA fragment of about 8 kilobase pairs (hereinafter, "kbp") containing the vector side was collected by using DNA collecting kit EASY TRAP Ver. 2 (~~DNA collection kit,~~ Takara Shuzo). The vector region fragment of the pVIC40 plasmid prepared as described above was self-ligated by using DNA Ligation Kit Ver. 2 (Takara Shuzo). This ligation reaction solution was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, Takara Shuzo). The cells were applied on the LB agar medium containing 20 mg/L of streptomycin and incubated overnight at 37°C. The colonies that appeared on the agar medium were each inoculated to the LB liquid medium containing 20 mg/L of streptomycin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture broth by the alkaline SDS method, and structure of each plasmid was confirmed by digestion with restriction enzymes to obtain pRS.

[0089] Then, a plasmid pRStac having the *tac* promoter was constructed from pRS

App'n Serial No.: 10/716,480

5

Att'y Docket No: US-102

according to the scheme shown in Fig. 1. The pRStac plasmid was constructed as follows. The pRS vector was digested with restriction enzymes EcoRI and PstI, and added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNAs were collected by ethanol precipitation and separated on 0.8% agarose gel. A DNA fragment of 8 kilobase pairs (henceforth abbreviated as "kbp") was collected by using DNA collection kit EASY TRAP Ver. 2 (~~DNA collection kit,~~ Takara Shuzo). On the other hand, the *tac* promoter region was amplified by PCR using the pKK223-3 plasmid (expression vector, Pharmacia) as a template and the primers shown in SEQ ID NOS: 7 and 8 (a cycle consisting of denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 60 seconds was repeated for 30 cycles). DNA polymerase Pyrobest-PYROBEST ~~DNA polymerase~~ (Takara Shuzo) was used for PCR. The DNA fragment containing the amplified *tac* promoter was purified by using PCR prep (Promega) and then digested at the restriction enzyme sites preliminarily designed in the primers, i.e., at EcoRI and EcoT22I sites. Then, the reaction mixture was added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNAs were collected by ethanol precipitation and separated on 0.8% agarose gel. A DNA fragment of about 0.15 kbp was collected by using DNA collecting kit EASY TRAP Ver. 2 (Takara Shuzo).

[0092] The *lysE* gene fragment was also amplified by PCR using chromosome extracted from the *Brevibacterium lactofermentum* 2256 strain (ATCC13869) as a template and the primers shown in SEQ ID NOS: 9 and 10 (denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 90 seconds). ~~Pyrobest~~-DNA polymerase PYROBEST (Takara Shuzo) was used for PCR. At this time, so that expression of the *lysE* gene is possible in a *Methylophilus* bacterium, the primers were designed so that nucleotides located 9-15 bp from the translation initiation codon of the *lysE* gene were replaced with a sequence that is known to function in a *Methylophilus* bacterium (Wyborn, N.R., Mills, J., Williamis, S.G. and Jones, C.W., Eur. J. Biochem., 240, 314-322 (1996)). The obtained fragment was purified by using PCR prep (Promega) and then digested with Sse8387I and SapI. The reaction mixture was added to a

phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected and DNAs were collected by ethanol precipitation and further collected from 0.8% agarose gel.

[0101] Subsequently, a mutation was introduced into the prepared pRSlysE by an *in vitro* mutation method using hydroxylamine. That is, a solution containing 250 mM potassium phosphate buffer adjusted to pH 6.0, 400 mM hydroxylamine solution adjusted to pH 6.0, each as a final concentration, and 2 µg of pRSlysE plasmid and made 200 µl water was prepared and incubated at 75°C for 2 hours or 3 hours. Subsequently, the pRSlysE plasmid was collected from this solution by using DNA collection kit EASY TRAP Ver. 2 (~~DNA collection kit~~, Takara Shuzo). Plasmids reacted with hydroxylamine for 2 hours and plasmids reacted with hydroxylamine for 3 hours were mixed to obtain an aggregate of pRSlysE plasmids introduced with mutations at various rates.

[0115] The *dapA*\* gene fragment was amplified by PCR using the known plasmid RSFD80 (see WO90/16042) containing that gene as a template and the primers shown in SEQ ID NOS: 11 and 12 (denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 60 seconds). Pyrobest-DNA polymerase PYROBEST (Takara Shuzo) was used for PCR. The resulting *dapA*\* fragment was purified by using PCR prep (Promega) and then digested with restriction enzymes *Sse8387I* and *XbaI*. The reaction mixture was added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNAs were collected by ethanol precipitation and separated on a 0.8% agarose gel to collect a DNA fragment of about 0.1 kbp.

[0117] In order to evaluate the effect of combining any of *lysE562*, *lysE564* or *lysE565* with *dapA*\*, plasmids pRSlysE562, pRSlysE564 and pRSlysE565 were constructed by insertion of the *dapA*\* gene. pRSlysE562, *lysE564* and *lysE565* prepared in Example 1 were digested with a restriction enzyme *SapI* and blunt-ended by using DNA Blunting Kit (Takara Shuzo). Furthermore, a pRSdapA plasmid was digested with restriction enzymes *EcoRI* and *SapI*, and a fragment of about 1 kbp having the tac promoter and the

App'n Serial No.: 10/716,480

7

Att'y Docket No: US-102

*dapA*\* region was separated on a 0.8% agarose gel and collected by using DNA collecting kit EASY TRAP Ver. 2 (Takara Shuzo). This fragment was blunt-ended as described above and ligated to each of the digestion products of the aforementioned pRSlysE562, *lysE564* and *lysE565* by using DNA Ligation Kit Ver. 2 (Takara Shuzo).

[0122] First, pRS-lysE564-Tc carrying the tetracycline resistance gene was constructed from pRSlysE564. The pRS-lysE564 plasmid was digested with a restriction enzyme *EcoRI* and added to a phenol/chloroform solution and mixed to terminate the reaction. The reaction mixture was centrifuged, and the upper layer was collected. DNAs were collected by ethanol precipitation, and the digested ends thereof were blunt-ended by using DNA Blunting Kit (Takara Shuzo). DNA fragments were separated on a 0.8% agarose gel, and a DNA fragment having about 9 kilobase pairs (henceforth abbreviated as "kbp") was collected by using DNA collection kit EASY TRAP Ver. 2 (~~DNA collection kit~~, Takara Shuzo).

[0123] Furthermore, the tetracycline resistance gene region was amplified by PCR using the pRK310 plasmid (Pansegrau et al., J. Mol. Biol. 239, 623-663 (1994) as a template and the primers shown in SEQ ID NOS: 13 and 14 (a cycle consisting of denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds and extension reaction at 72°C for 60 seconds was repeated for 30 cycles). ~~Pyrob~~est-DNA polymerase PYROBEST (Takara Shuzo) was used for PCR. The amplified DNA fragment containing the tetracycline resistance gene region was purified by using PCR prep (Promega), and then DNAs were collected by ethanol precipitation, blunt-ended and phosphorylated by using Blunting Kination Ligation Kit TaKaRa BKL Kit (~~Blunting Kination Ligation Kit~~, Takara Shuzo), added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNAs were collected by ethanol precipitation and separated on a 0.8% agarose gel. A DNA fragment of 1.5 kbp was collected by using DNA collecting kit EASY TRAP Ver. 2 (Takara Shuzo).